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13. ABSTRACT *(Maximum 200)*

We have constructed a series of artificial chimeric genes each composed of a tumor-associated CD44 alternative splicing unit (ASU) which includes alternatively spliced exons and flanking introns and exons and the cytosine deaminase (CD) gene. The selective expression of active CD44/CD chimeric protein potentially enables tumor-specific killing via the RNA metabolism of alternative splicing following administration of the prodrug 5-FC. Insertion of the CD44 transmembrane region between 5'CD44 encoding exons and 3'-CD cDNA anchors the chimeric protein to the cell membrane while partitioning CD44 to the extracellular and CD to the intracellular compartments, respectively and results in no significant loss of CD enzymatic function. This modification makes it possible to introduce specific therapeutic receptor or antigen sites located outside the membrane allowing for recognition of cancer cells surviving the toxicity of the 5-FU anabolite. Concluions: (1) We have generated a variety of CD44/CD fusion protein DNA constructs which include CD44 cDNA+CD and CD44 (ASU)+CD. (2) The introduction of CD44 transmembrane domain into the chimeric proteins potentially allows for the combination of CEPT and immunotherapy in a single vector. (3) RSV promoter-driven CD or CD44/CD plasmids rendered T47D breast cancer cells sensitive to 5-FC. Expression efficiency of the proteins in breast cancer cells was further improved by replacement of the pBK-RSV vector with pBK-CMV which has stronger inherent activity in breast cells.

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David L Cagen 9-10-97

PI - Signature Date

TABLE OF CONTENTS

(5) INTRODUCTION: Chimeric Enzyme/Prodrug Therapy (CEPT) as Novel Gene Therapy for Breast Cancer

CD44 is a heterogeneous cell adhesion molecule which exhibits a variety of isoforms that are generated from a single gene by alternative splicing of messenger RNA.[1,2] Recent reports showed that roughly 80% of systemic tumors metastatic to the brain exhibit CD44 isoform expression.[3] The correlation between CD44 variant isoform expression and metastases of breast carcinomas, which contribute to a considerable fraction of systemic tumors metastatic to the brain,[4] provides a pathway to developing a potential therapeutic intervention of metastatic brain cancer. Based on the hypothesis that selective killing of metastatic cancer cells may be achieved by exploiting the differences between normal and neoplastic cells to alternatively splice CD44 pre-mRNA, we have tried to utilize tumor-mediated CD44 alternative splicing signals as an additional gene therapeutic control element.[5] We constructed a series of artificial chimeric genes each composed of a tumor-associated CD44 alternative splicing unit (ASU) which includes alternatively spliced exons and flanking introns and exons and a cytosine deaminase (CD) gene. CD is a bacteria-originated protein which converts the relatively nontoxic prodrug 5-fluorocytosine (5-FC) to the toxic anti-cancer drug 5-fluorouracil (5-FU) anabolite.[6-8] The selective expression of active CD44/CD chimeric protein with CD activity enables tumor cell killing via tumor-specific alternative splicing pathways following administration of the prodrug 5-FC.

In our initial steps, we developed a novel *in vitro* assay system for rapid analyses of potentially therapeutic CD44 alternative splicing minigene constructs. We also cloned the *E. coli* CD gene, fused its enzymatically active domain to alternatively spliced CD44 exons (CD44 cDNA/CD) and demonstrated that CD44(v8v9)/CD chimeric protein with spliced variant exons 8 and 9 retain CD enzymatic activity.[5] To further our efforts toward the development of novel gene therapeutic approaches, we have recently generated various second generation CD44/CD DNA constructs. These constructs all contained a transmembrane (TM) domain of CD44 gene and downstream CD cDNA. Additionally, various standard CD44 cDNA fragments or CD44 genomic fragments with ASUs were amplified and ligated in translational reading frame with downstream TM+CD. All the inserts were cloned into vectors capable of both bacterial and mammalian expression. The introduction of a CD44 TM domain anchors each chimeric protein to the mammalian cell membrane with the CD domain on the cytoplasmic side of the membrane. This modification allows introduction of additional specific receptor or antigen sites located on the extra cellular membrane allowing for recognition of cancer cells survived the toxicity of 5-FU anabolite through immuno therapeutic or other gene therapeutic approaches.

(6) BODY: EXPERIMENTAL PROCEDURES, RESULTS AND DISCUSSION

Construction of bacterial and mammalian expression vectors

Figure 1 and 2 showed the structures of CD cDNA and chimeric CD44/CD fusion protein cDNA constructs. Various CD44 cDNA fragments were fused in translational reading frame with the cloned CD cDNA. The 5 constructs were: A. the CD44(LD+E2-5,16,17+TM)+CD construct which contained a PCR amplification cassette of the standard CD44 (CD44s) cDNA with the 5' untranslated leader sequence and adjacent hydrophobic amino acid residues in exon one of the secretory peptide sequence (LD), exons 2, 3, 4, 5, 16, 17 and 18 with the transmembrane domain (TM) sequence; B. the CD44(LD+E2+TM)+CD construct with a PCR amplification cassette of CD44 cDNA with LD and exon 2, followed by a TM PCR amplification cassette; C. the CD44(LD+TM)+CD construct with two PCR amplification cassettes, LD and TM; D. the CD44(TM)+CD construct with TM PCR amplification cassette; and E. wild-type CD only. The CD44 cDNA fragments above were amplified from pCDM8/CD44H plasmid which contained full length of CD44s cDNA (Fig 1). Figure 3 showed the structures of recombinant DNAs with CD44 ASUs fused in translational frame to CD cDNA. These recombinants included: A. CD44(v8-v9-v10+TM)+CD construct with CD44 variant exons 8, 9 and 10, introns 8 and 9, and TM; B. CD44(v8-v9+TM)+CD construct with CD44 variant exons 8 and 9, intron 8 and TM; C. CD44(v9-v10+TM)+CD construct with CD44 variant exons 9 and 10, intron 9 and TM. The CD44 genomic DNA fragments above were amplified from normal cell genomic DNA using PCR. To facilitate the preparation and cloning of the constructs, certain restriction enzyme sites and 5'-end additional flanking nucleotides were also incorporated in the forward and reverse primers.

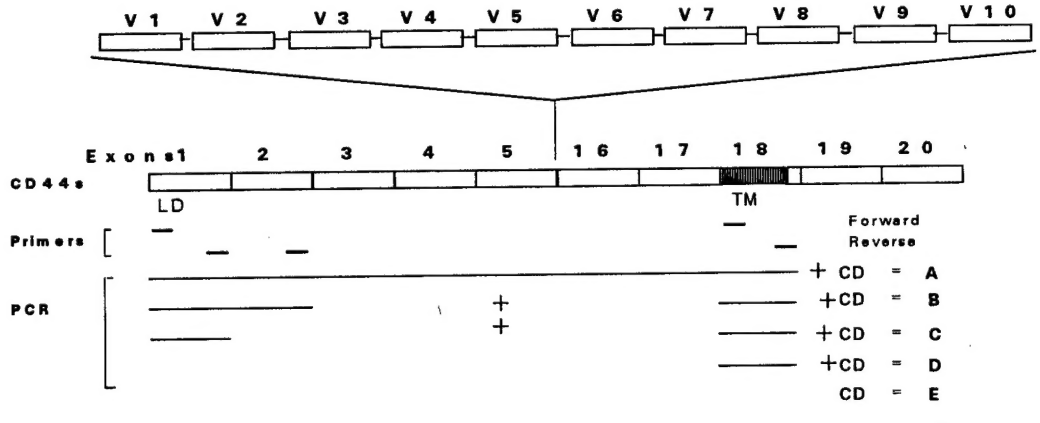


Fig. 1 The inserts of the vector constructs (A-E) consisting of either CD44 cDNA+ CD or CD alone. The constructs A-D all contain TM domain.

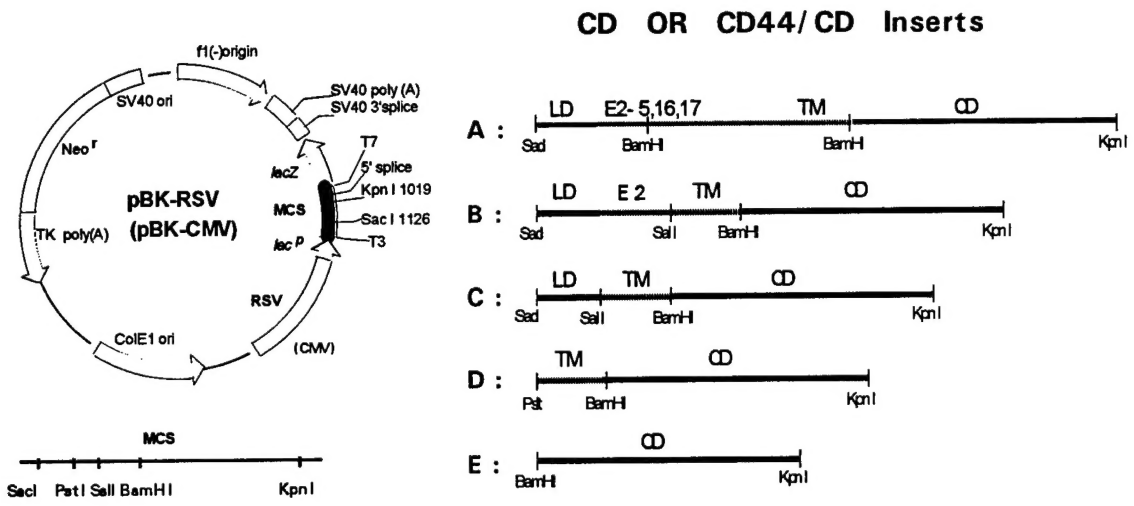


Fig. 2 Cloning of the inserts into the multiple cloning sites (MCS) of pBK-RSV and pBK-CMV vectors (Stratagene).

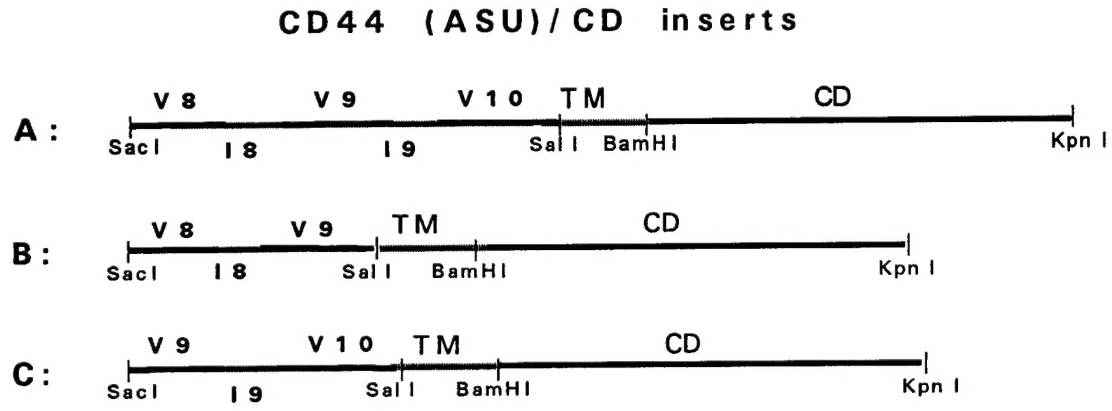


Fig. 3 Structures of CD44 (ASU)/CD inserts. The inserts were cloned into pBK-CMV vectors. V8, V9 and V10: variant exons; I8 and I9: introns.

PCR was performed in a 50ul reaction volume in a Perkin-Elmer Cetus 480 thermal cycler for 35 cycles consisting of 94°C for 45 sec and 72°C for 3 min. Following appropriate restriction enzyme digestions and ligations, the corresponding PCR amplicons were joined together in translational reading frame, and all the inserts (Fig. 2A-E, and Fig. 3 A-C) were cloned into the complementary restriction endonuclease sites located in the MCS of pBK-RSV vector (Stratagene) with *lacZ* and RSV LTR promoters, and/or in the MCS of pBK-CMV vector (Stratagene) *lacZ* and CMV promoters, both suitable for prokaryotic and eukaryotic expressions.

Transformation and CD enzymatic assay

All the ligated plasmid constructs were transfected into *E. coli* strain XL-1 cells by electroporation using a Gene Pulser (Bio-Rad) and amplified in overnight cultures of the transformed cells under tetracycline and kanamycin selective pressure. After screening by restriction enzyme digestion and sequencing of isolated plasmid DNA, the right clone with the designed plasmid was selected and grown in 5ml LB media to a concentration of 10^9 bacterial cells/ml. Cells lysate was prepared as previously described. The ability of the cell lysate to deaminate cytosine was determined, by the method of West and O'Donovan[6] with modification, in 1 ml assay mixture containing 50mM Tris-HCl (pH7.3), 100ug cell extract protein (protein concentrations were determined using a Bio-Rad protein determination kit), and 2.5mM cytosine. Decreases in absorbance at 285nm were measured over time (Fig. 4).

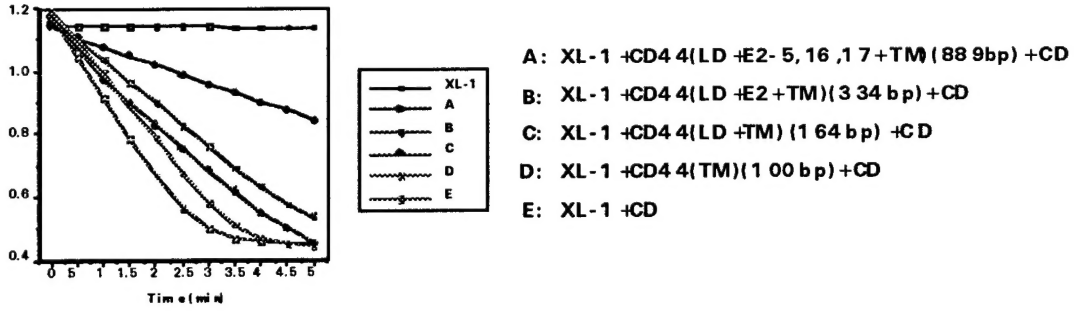


Fig. 4 CD enzymatic activities in the cell lysates of XL-1 bacterial strains without or with the CD44/CD or CD plasmids (Fig. 2).

Expression and activity of chimeric CD44/CD proteins expressed in *E. coli* bacteria

The bacterial clones were grown under proper antibiotic selection pressure. Following induction by IPTG, protein lysates were analyzed by SDS-polyacrylamide gel electrophoresis. Coomassie bright blue staining showed bacterial expression of wild-type CD (52KDa) and larger molecular weight CD44/CD chimeric fusion proteins (data not shown).

Enzymatic verification of CD activity showed that wild-type CD protein in the cell lysate of bacterial clone with plasmid containing wild-type CD gene displayed extensive CD activity which effectively converted cytosine into uracil resulting in rapid decrease in absorbance at 285nm (Fig. 4E). The chimeric CD44/CD fusion proteins continued to exhibit substantial enzymatic activity even following the addition of up to 889 bp DNA fragment 5' in frame to CD gene (Fig. 4A-D). Meanwhile, the inclusion of transmembrane sequences to tether CD to the cell membrane apparently have little affect on enzymatic activity.

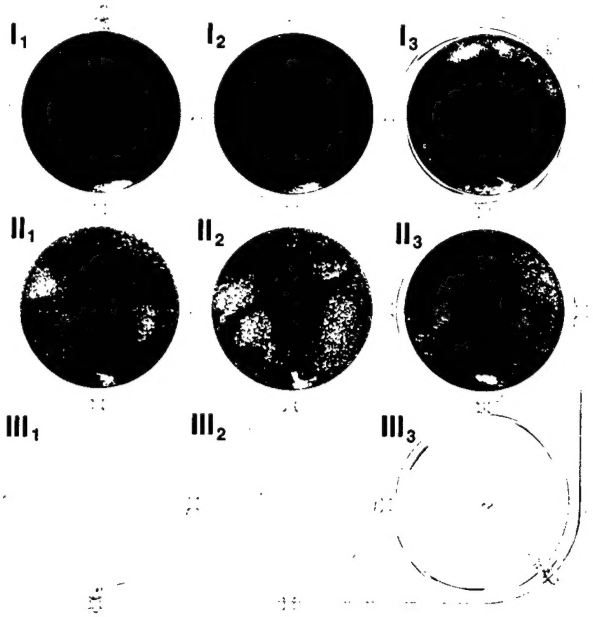
Mammalian cell stable gene transfection

All plasmid constructs with RSV or CMV promoters were then transferred into human breast cell line T47D using a mammalian transfection kit (Stratagene) according to the manual provided by the manufacturer The transfected cells were splited at the ratio of 1:10 and then exposed to selection culture medium with 250ug/ml G418. G418-resistant cell colonies were obtained and grown up after exposure of the cells to the selection medium for 2-4 weeks.

***In vitro* 5FC or 5FU sensitivity assay**

Cells containing RSV promoter-driven DNA constructs were plated at a density of 10^4 cells/well in 6-well plates (Corning) and incubated with new medium containing various concentrations of 5FC (0, 0.04, 0.2 and 1 mM) and 5FU (0, 0.04, 0.2 and 1 mM) at 37°C for 7-14 days. The cells were then fixed with 5% formaldehyde at room temperature for 2 hours, stained with 0.5% crystal violet for 20 minutes, rinsed with deionized water and air dried (Fig. 5).

The stably transfected breast cancer cell line T47D with various constructs driven by RSV promoter were incubated in medium containing different concentrations of 5FC and 5FU. The results showed that while up to 1mM 5FC remained nontoxic to intact T47D cells (Fig. 5A), the transfected cells expressing either wild-type CD or chimeric CD44/CD fusion proteins exhibited markedly reduced survival in 5-FC (Fig. 5B-E). Meanwhile, a clear dose-response relationship between 5-FC concentration and cell killing was observed. Notably, the sensitivities of cells expressing CD44(TM)+CD or CD44(E2+TM)+CD to 5FC seemed to be similar or even a little higher than that of cells expressing wild-type CD. The inconsistency of this observation with the results of the CD enzymatic activity assays in bacteria may be explained if the TM-CD protein, through attachment to the cell membrane makes it less susceptible to cellular metabolism, effectively increasing its cytoplasmic half life.



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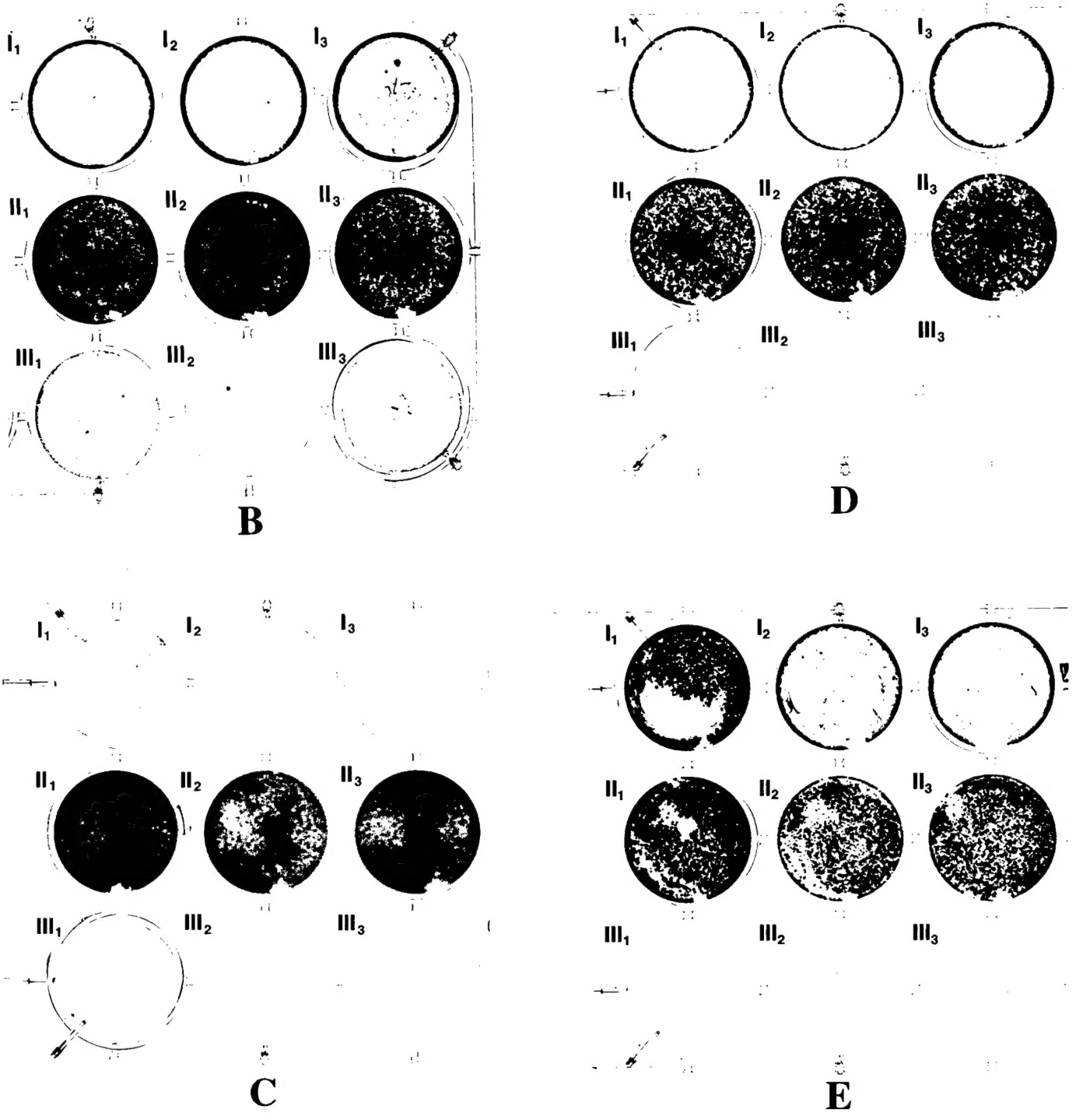

Fig. 5 Staining of surviving cells after treatment with 5-FC, normal medium and 5-FU. The untransfected cells were killed by 5-FU but not 5-FC (A). The cells expressing wild-type CD (B), CD44(TM)+CD (C), CD44(E2+TM)+CD (D) and CD44(E2-5, 16,17+TM)+CD (E) exhibited increased sensitivities to 5-FC. A clear dose-response relationship between 5-FC concentration and cell killing was observed. Rows I, II and III correspond to treatment of the cells with medium+5-FC, medium only and medium+5-FU respectively. Columns 1, 2 and 3 correspond to addition of 0.04, 0.2 and 1mM 5-FC or 5-FU respectively.

To verify the above hypotheses, we have started to make anti-CD protein antibody, which is necessary to compare the stabilities of the chimeric proteins with that of wild-type CD and to detect the localizations of these proteins, with antigen (CD protein) prepared using bacterial protein expression and purification system. It was also noted that cells located at the edges of wells still survived even when exposed to 5-FC for 14 days. In order to increase the expression efficiency of the proteins, we have recently replaced the pBK-RSV plasmid with pBK-CMV containing stronger promoter and transfected T47D cells with the new constructs.

Bacterial CD protein expression and purification

Bacterial CD protein expression and purification were carried out using QIAexpressionist system (QIAGEN). CD cDNA was cloned into bacterial vector pQE30 and transfected into host strain M15 as described above. The expressed protein using such a vector/bacteria system contains an upstream 6XHis affinity tag which strongly binds to Ni-NTA resin at pH8.0 allowing for purification of the protein by affinity chromatography. Briefly, a single colony with the expression plasmid was incubated in 20ml LB media with 100ug/ml ampicillin and 25ug/ml kanamycin at 37°C until the A600 reached 0.8. After 2mM IPTG induction for 5 hours, the cells were harvested by centrifugation, resuspended in sonication buffer (50mM Na-phosphate pH8.0 and 300mM NaCl), and sonicated on ice until the A260 of the lysate reached a maximum. The CD protein was then binded to Ni-NTA resin in sonication buffer, washed with sonication buffer and then wash buffer (50mM Na-phosphate pH6.0, 300mM NaCl and 10% glycerol), and eluted with 250mM imidazole in wash buffer. The purified CD protein was allocated in proper concentration and sent to Atlantic Antibodies company for preparation of rabbit anti-CD antibody.

(7) CONCLUSIONS

(1) We have generated a variety of CD44/CD fusion protein DNA constructs which include CD44 cDNA+CD and CD44 (ASU)+CD.

(2) The introduction of CD44 transmembrane domain into the chimeric proteins potentially allows for the combination of CEPT and immunotherapy in a single vector.

(3) RSV promoter-driven CD or CD44/CD plasmids rendered T47D breast cancer cells sensitive to 5-FC. Expression efficiency of the proteins in breast cancer cells was further improved by replacement of the pBK-RSV vector with pBK-CMV which has stronger inherent activity in breast cells.

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